Hepatobiliary & Pancreatic Diseases International 18 (2019) 255–265

Contents lists available at ScienceDirect

Hepatobiliary & Pancreatic Diseases International

journal homepage: www.elsevier.com/locate/hbpd

Original Article/Liver

Sphingosine kinase 1 knockout alleviates hepatic ischemia/reperfusion injury by attenuating inflammation and oxidative stress in mice

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ARTICLE INFO

Article history:
Received 26 October 2018
Accepted 1 April 2019
Available online 5 April 2019

Keywords:
Ischemia/reperfusion injury
Sphingosine kinase 1
Inflammation
Oxidative stress

ABSTRACT

Background: Hepatic ischemia/reperfusion (I/R) injury remains a significant problem in clinical practice. Sphingosine kinase 1 (SphK1) phosphorylates sphingosine to sphingosine-1-phosphate (S1P) which participates in multiple bioactive processes. However, little is known about the role of SphK1 in hepatic I/R injury. This study aimed to investigate the effect of SphK1 knockout on liver I/R injury and to explore underlying mechanisms.

Methods: SphK1 knockout and wild type mice were subjected to 70% partial hepatic I/R. Serum alanine aminotransferase was determined to indicate the degree of liver damage. Hematoxylin-eosin staining and TUNEL assay were used to assess histological changes and hepatocellular apoptosis, respectively. Immunohistochemistry was performed to detect the expression and translocation of phosphorylated p65 and signal transducer and activator of transcription 3 (STAT3). Western blotting was used to determine the expression of S1P receptor 1 (S1PR1), phosphorylated p65 and STAT3. Real-time PCR was used to demonstrate the changes of proinflammatory cytokines. Oxidative stress markers were also determined through biochemical assays.

Results: SphK1 knockout significantly ameliorated I/R-induced liver damage, mitigated liver tissue necrosis and apoptosis compared with wild type control. I/R associated inflammation was alleviated in SphK1 knockout mice as demonstrated by attenuated expression of S1PR1 and reduced phosphorylation of nuclear factor kappa B p65 and STAT3. The proinflammatory cytokines interleukin-1β, interleukin-6 and tumor necrosis factor-α were also inhibited by SphK1 genetic deletion. The oxidative stress markers were lower in SphK1 knockout mice after I/R injury than wild type mice.

Conclusions: Knockout of SphK1 significantly alleviated damage after hepatic I/R injury, possibly through inhibiting inflammation and oxidative stress. SphK1 may be a novel and potent target in clinical practice in I/R-related liver injury.

Introduction

Hepatic ischemia/reperfusion (I/R) injury is a pathologic process that occurs in various clinical settings, including surgical procedures, liver transplantation, low flow status and other pathologic conditions [1]. Hepatic I/R injury may lead to acute or chronic organ failure and cause morbidity and mortality [2]. The damage caused by hepatic I/R injury are triggered by transient blood flow deprivation and restoration. The temporary ischemic insult activates proinflammatory reaction and increases reactive oxygen species (ROS), which further damage the liver [3,4]. Though numerous basic and clinical studies have demonstrated underlying mechanisms and experimental therapies, ideal therapeutic approach or drug for hepatic I/R injury is still not satisfactory [5]. Therefore, further studies to shed light on the molecular mechanisms and potential efficient therapies of liver I/R injury is urgently needed.

Sphingosine-1-phosphate (S1P) is a bioactive signaling sphingolipid metabolite that mediates cellular proliferation, migration, differentiation, immune reaction and inflammation [6]. S1P is formed intracellularly by the phosphorylation of sphingosine. This process is catalyzed by two sphingosine kinases: sphingosine

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https://doi.org/10.1016/j.hbpd.2019.04.001
1499-3872/

kinase 1 (SphK1) and sphingosine kinase 2 (SphK2). After the formation, S1P is exported and interacts with five G protein coupled receptors (sphingosine-1-phosphate receptors 1–5, S1PR1-5). The signaling of the receptors mediates various physiologic and pathologic processes, including inflammation and reactive oxygen species (ROS) production [6,7]. S1P mediated transduction through S1PR1 leads to the activation of master inflammatory transcription factors nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) through a positive feedback amplification loop [8]. The activation of these transcription factors subsequently causes the expression of various toxic proinflammatory cytokines including interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α). Sphingosine kinases and S1P signaling also increases cyclooxygenase-2, nitric oxide synthase (NOS), adhesion molecules and the release of ROS, which mediate the process of oxidative stress [9–12].

Dysregulation of sphingosine metabolism has been shown to participate in I/R injury. Myocardial infarction markedly increases plasma S1P and the level of S1P predicts the severity of disease [13]. Vessey et al. reported that SphK2 knockout sensitizes mice to myocardial I/R injury and diminishes responsiveness to preconditioning [14]. Similarly, adenoviral transfection of SphK1 and S1P treatment demonstrated protective effect against heart I/R damage [15,16]. In contrast, renal I/R injury are alleviated by S1PR3 knockout [17]. For hepatic I/R injury, chemical inhibition of SphK2 shows protective effect through the improvement of mitochondrial function [12] and suppression of inflammation [18]. S1P attenuates both hepatic and renal injury induced by hepatic I/R in mice model [19]. Accordingly, signaling through S1PR2 triggers hepatic wound healing [20]. Exosomal transfer of SphK2 also enhances liver repair after I/R injury or hepatectomy [21,22]. These inconsistent results indicate the role of S1P metabolism and signaling may be organ, kinase and receptor specific [12].

As a major sphingosine kinase, the role of SphK1 in hepatic I/R injury has not been reported. The present study was to explore the effect of SphK1 knockout on liver I/R injury and the potential mechanisms underlying these effects.

Methods

Animals and genotyping

Heterozygous SphK1 knockout mice (SphK1$^{+/-}$ mice) were obtained from the Jackson Laboratory (kindly gifted by Dr. Richard L. Proia). The mice were bred according to the manufacturer's protocol in the Experimental Animal Center of the Drum Tower Clinical College of Nanjing Medical University. The animals were given free access to tap water and standard mouse diet. This experiment was approved by the Ethics Committee of Drum Tower Clinical College of Nanjing Medical University.

Genotyping of offspring mice were performed as previously described [23]. Briefly, total genomic DNA was obtained by tail tip biopsy and further purification. DNA free water was used as negative control and DNA from wild type C57BL/6 mice was used as positive control. Polymerase chain reaction (PCR) was performed using primers as follows: primer 1, 5′-TGT CAC CCA TGA ACC TGC TGT CCC TGC ACA; primer 2, 5′-AGA AGG CAC TGG CTC CAG AGG AAC AAG; primer neo, 5′-TCG TGC TTT ACG GTA TCG CCG CTC CCG ATT (94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min). PCR yielded a DNA fragment of about 300 bp from wild-type allele and a fragment of about 350 bp from knockout allele.

I/R injury model

After genotyping, 8–10 weeks homozygous knockout mice (SphK1$^{-/-}$ mice) and wild type littermate control mice (wild type mice) were used in the I/R model ($n = 10$ for each group). The model of 70% partial hepatic I/R was established as previously described [24,25]. Briefly, a midline laparotomy was performed under isoflurane anesthesia. Portal triad to the left lateral and median lobes was occluded using atraumatic microvascular clip for 90 min. During the ischemia phase, the incision was covered by 0.9% saline moistened gauze. Then the clip was removed for reperfusion and about 500 μL sterile saline was administered into the peritoneal cavity to replenish fluid loss during surgical procedure. The abdomen was then closed with continuous silk suture. The mice were sacrificed at 6 h or 12 h after reperfusion and blood and liver tissue samples were obtained for further analysis. Sham operation with the same procedure without portal triad occlusion was performed as control.

Real-time PCR

RNA was extracted, purified and converted to cDNA with RNAisoPlus kit and PrimeScript RT Master Mix (Takara Bio Inc., Naha, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was performed using a ViiA 7 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Rockford, IL, USA) with SYBR Premix EX Taq II reagent (Takara Bio Inc.).

The primers used in real-time PCR were as follows: SphK1, forward 5′-ACA GTG GGC ACC TTC TTT C, reverse 5′-CTT CTG CAC CAG TGT AGA GGC; IL-1β, forward 5′- GCA ACT GTT CCT GAA CTC AAC T, reverse 5′- ATC TTT TGG GGT CCG TCA ACT; IL-6, forward 5′-TGA TTG TAT GAA CAA CGA TGA TGC, reverse 5′- GGA CTC TGG CTT TGT CTT TCT TGT; TNF-α, forward 5′- CAC GCT CTT CTG TCT ACT GAA C, reverse 5′- ATC TGA GTG TGA GGG TCT GG; GAPDH, forward 5′- CAA CTA CAT GGT CTA CAT GTT C, reverse 5′- CAC CAG TAG ACT CCA CGA C.

Serum alanine aminotransferase (ALT) measurement

Blood was obtained immediately after sacrifice. The serum concentration of ALT was measured using an ALT detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol.

Histology and immunohistochemistry

Liver tissue specimens were fixed with 4% buffered paraformaldehyde and embedded in paraffin. Slices of tissue were stained with hematoxylin and eosin (HE) for the assessment of tissue damage. The areas of necrosis were identified and the percentages of necrotic area was quantified using ImageJ 1.8.0 software (NIH, http://imagej.nih.gov/ij).

For immunohistochemistry analysis, the sectioned slices were stained for phosphorylated STAT3 and phosphorylated p65. Briefly, slices were deparaffinized, rehydrated and stained using monoclonal primary antibodies from Cell Signaling Technology (Danvers, MA, USA). A horseradish peroxidase (HRP)-conjugated secondary antibody and diaminobenzidine (DAB) were used to detect immunostaining. The slices were counterstained with hematoxylin. Integrated optical density (IOD) of phosphorylated STAT3 specific staining was measured using ImageJ software and nuclear positive rate of phosphorylated p65 was counted. For terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, a commercial TUNEL kit (Beyotime, Nantong, China) was used. Tissue slices were incubated with proteinase K and then washed with phosphate buffered saline (PBS). 0.3% hydrogen peroxide (H_2O_2) was used to block innate peroxidase activity. After incubation with TUNEL reaction solutions, the slices were washed and incubated with HRP-conjugated streptavidin. Finally,

DAB solution was incubated and positive cells per high-power field were calculated.

Western blotting

Tissue lysates were prepared using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime) supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland). Total protein concentration was determined by BCA reagent following the manufacturer's instruction (Thermo Fisher Scientific). Equal amount of soluble proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), proteins were detected by incubation with primary antibodies (phosphorylated STAT3, phosphorylated p65 and GAPDH, Cell Signaling Technology; S1P receptor 1, S1PR1, Abcam, Cambridge, MA, USA; SphK1, Proteintech Group Inc., Rosemont, IL, USA) followed by HRP-conjugated secondary antibodies. Enhanced chemiluminescence (ECL) reagent (Millipore) was applied to the membranes and specific protein bands were visualized by FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA, USA).

Oxidative stress-related assays

The activities or concentrations of liver myeloperoxidase (MPO), malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD), H_2O_2, catalase (CAT), nitric oxide (NO), total nitric oxide synthase (NOS) activity and inducible nitric oxide synthase (iNOS) activity were determined using commercial biochemical reagent kits (Nanjing Jiancheng Bioengineering Institute). Total protein concentration of liver specimens was determined using Coomassie blue assay.

Statistical analysis

The results were expressed as means ± standard deviation (SD). Differences among multiple groups were determined using one-way analysis of variance (ANOVA), followed by a post-hoc multiple comparison. Comparisons between two groups were performed using unpaired Student's *t*-test. A P value < 0.05 was considered statistically significant.

Results

Genotyping of wild type and SphK1 knockout mice

Fig. 1A showed that PCR of homozygous SphK1 knockout mice (SphK1$^{-/-}$ mice, No. 4 and 11) yielded a single DNA fragment of about 350 bp, while a single fragment of about 300 bp was generated from wild type littermate control mice (SphK1$^{+/+}$, No. 1, 3, 5, 7, 8, 10, 12, 13), which was in accordance with normal C57BL/6 mice positive control (B6). Heterozygous mice (SphK1$^{+/-}$, No. 2, 6, 9) yielded both of the ~300 bp and ~350 bp bands and negative control (NC) produced no band [23]. The protein levels of SphK1 in wild type and SphK1$^{-/-}$ mice were shown in Fig. 1B, and SphK1 knockout completely abolished the expression of SphK1.

Expression of SphK1 and serum ALT levels after hepatic I/R injury

After 90 min of 70% hepatic I/R in wild type mice, the expression of SphK1 was increased by 2.888 ($P < 0.001$) and 4.675 ($P < 0.001$) folds as compared with sham operation control (Fig. 1C). SphK1 knockout significantly reduced the level of ALT after 6 h and 12 h of reperfusion compared with wild type mice (wild type versus SphK1$^{-/-}$, I/R 6 h: 12,377 ± 1540 U/L versus 6907 ± 452 U/L, $P < 0.01$, Fig. 1D; I/R 12 h: 6395 ± 441 U/L versus 1513 ± 398 U/L, $P < 0.001$, Fig. 1E).

SphK1 knockout attenuated hepatic histopathological damage after I/R injury

As demonstrated in Fig. 2A, sham operation groups of both wild type and SphK1$^{-/-}$ mice exhibited normal liver cellular structure. I/R caused obvious areas of necrosis, hemorrhage and inflammatory cell infiltration in liver tissue of wild type. The areas

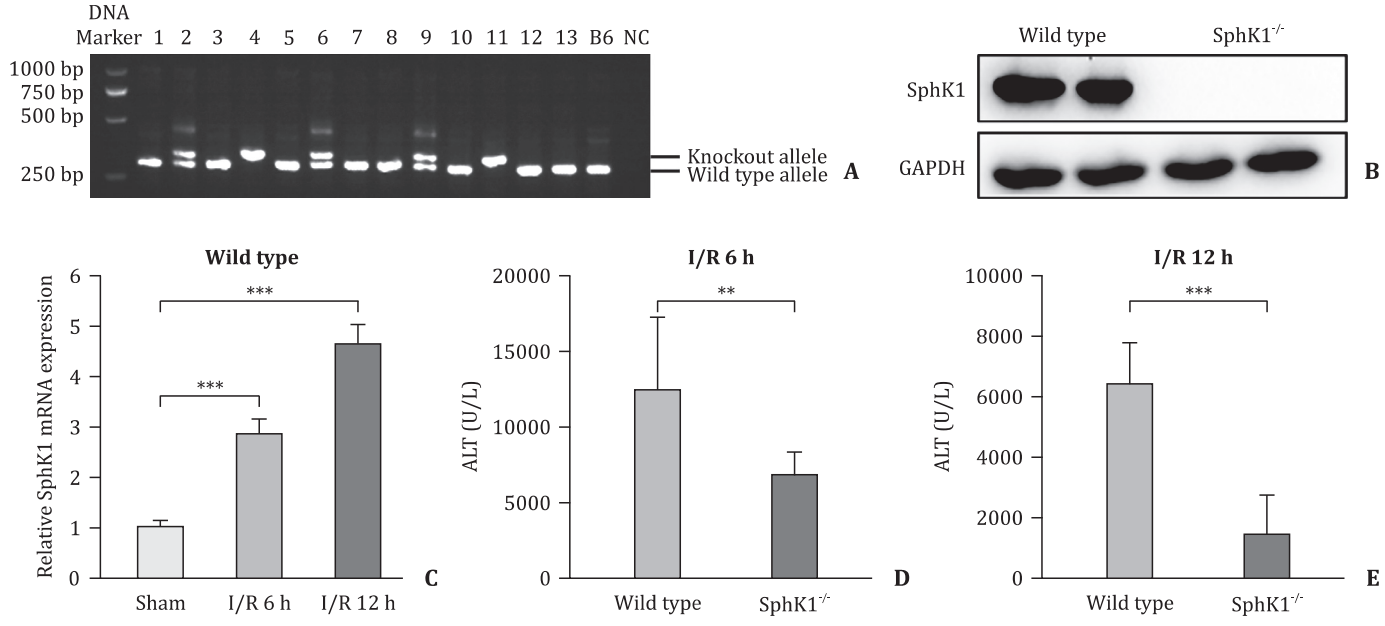

Fig. 1. Genotyping of mice and the determination of SphK1 expression and serum ALT levels after hepatic I/R injury. **A:** Results of PCR genotyping of SphK1 and wild type mice; **B:** Detection of SphK1 in liver tissue by Western blotting; **C:** Expression of SphK1 after I/R injury detected by real-time PCR ($n = 4$ for each group, this result is representative for three repeats of real-time PCR experiments); **D:** ALT level after ischemia and reperfusion of 6 h ($n = 10$ for each group); **E:** ALT level after ischemia and reperfusion of 12 h ($n = 10$ for each group). **: $P < 0.01$; ***: $P < 0.001$. B6: wild type C57BL/6 control; NC: negative control; I/R; ischemia/reperfusion; ALT: alanine aminotransferase; PCR: polymerase chain reaction.

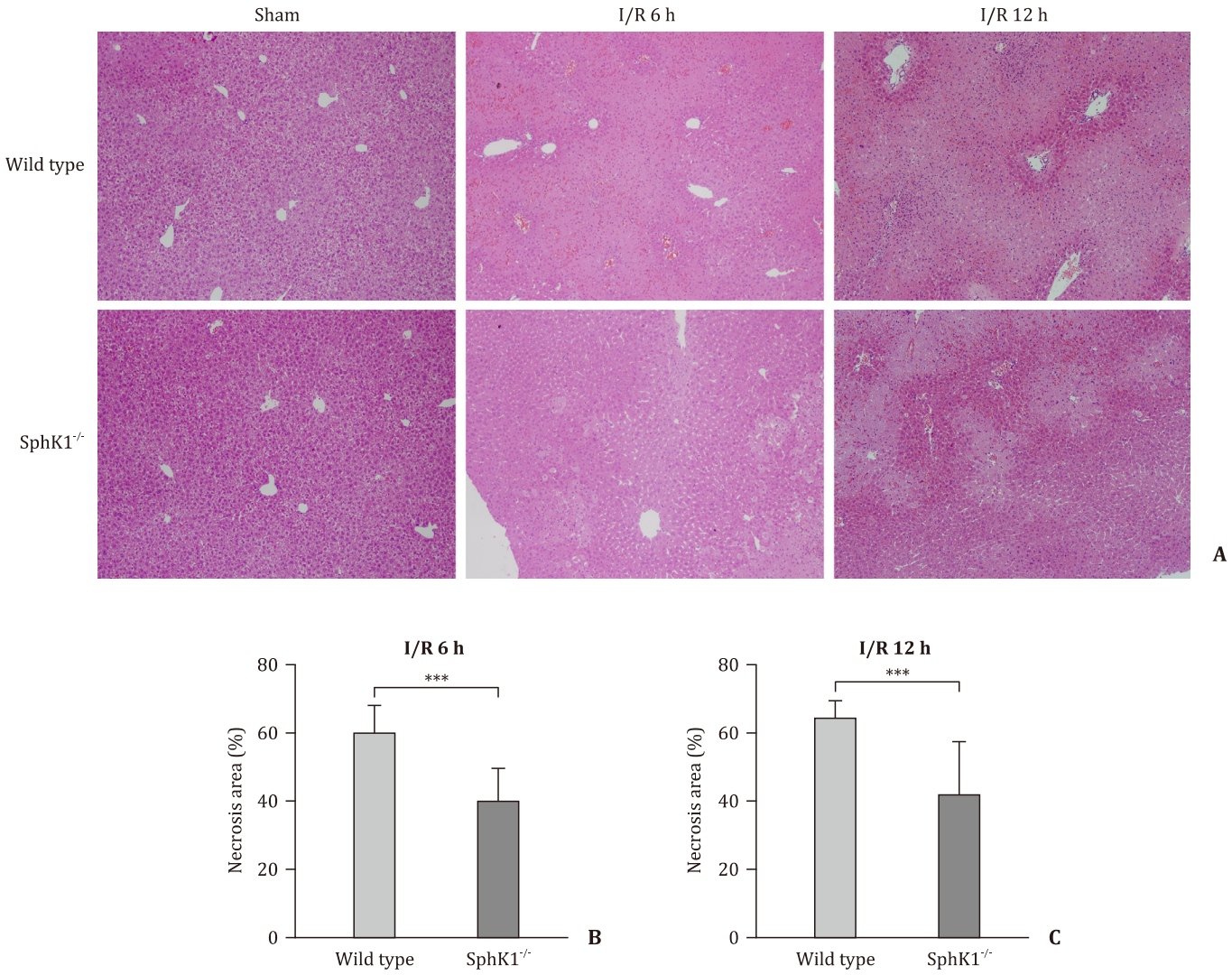

Fig. 2. Histologic changes after I/R injury. **A:** Histologic changes after reperfusion of 6 h and 12 h ($n = 10$ for each group, light microscope, HE staining, original magnification × 100); **B:** Quantitation of necrosis area after reperfusion of 6 h ($n = 10$ for each group); **C:** Quantitation of necrosis area after reperfusion of 12 h ($n = 10$ for each group). ***: $P < 0.001$. I/R: ischemia/reperfusion; HE: hematoxylin and eosin.

of necrosis was significantly milder in SphK1$^{-/-}$ mice (6 h, wild type versus SphK1$^{-/-}$, 59.6% ± 2.8% versus 40.2% ± 3.0%, $P < 0.001$, Fig. 2B; 12 h, wild type versus SphK1$^{-/-}$, 64.0% ± 1.7% versus 42.2% ± 4.8%, $P < 0.001$, Fig. 2C).

SphK1 knockout attenuated hepatocyte apoptosis

Fig. 3A showed that sham operation groups of wild type and SphK1$^{-/-}$ mice exhibited sparse TUNEL positive cell. After 6 h of reperfusion, TUNEL positive cells indicating apoptotic hepatocytes were intensively visualized in wild type mice [272.4 ± 7.9 per high power field (HPF)] whereas SphK1 knockout significantly attenuated hepatocyte apoptosis (176.2 ± 29.0 per HPF, $P < 0.01$, Fig. 3B). Similar results were demonstrated in 12 h reperfusion where TUNEL positive cells were 282.7 ± 8.1 per HPF in wild type mice and 198.9 ± 18.8 per HPF in SphK1$^{-/-}$ mice ($P < 0.001$, Fig. 3C).

SphK1 deletion inhibited liver I/R-induced inflammation

Immunohistochemistry (Fig. 4A) indicated that the phosphorylation of STAT3 was significantly induced by I/R of 6 h in wild type mice (IOD 37,990 ± 6088) (Fig. 4B). This induction of STAT3 activity slightly reduced after 12 h of reperfusion (IOD 30,571 ± 4877) (Fig. 4C). SphK1 knockout significantly alleviated the activation of STAT3. At 6 h after reperfusion, the IOD of phosphorylated STAT3 was 5423 ± 1384 in SphK1 knockout mice as compared with wild type mice ($P < 0.001$, Fig. 4B), and was 12363 ± 993 at 12 h after reperfusion ($P < 0.01$, Fig. 4C).

Fig. 5A showed that I/R significantly increased the nuclear positive rate of phosphorylated p65, which peaked at 6 h after reperfusion (32.7% ± 0.4%) and partly dropped at 12 h (13.3% ± 0.7%) in wild type mice. In SphK1$^{-/-}$ mice, the nuclear translocation of phospho-p65 was decreased. The nuclear positive rate of phosphorylated p65 was 19.9% ± 1.7% at 6 h after reperfusion ($P < 0.001$ compared with wild type, Fig. 5B) and 8.3% ± 1.0% at 12 h after reperfusion ($P < 0.001$ compared with wild type, Fig. 5C).

Fig. 6A, showed that SphK1 knockout alleviated the phosphorylation of STAT3, p65, and inhibited the activation of S1PR1. Quantitative real-time PCR demonstrated that wild type and SphK1 knockout mice had similar baseline expressions of IL-1β, IL-6 and TNF-α. I/R significantly elevated the expressions of these cytokines in wild type mice. The deletion of SphK1 significantly inhibited IL-1β ($P < 0.001$, Fig. 6B), IL-6 ($P < 0.01$, Fig. 6C) and TNF-α ($P < 0.001$,

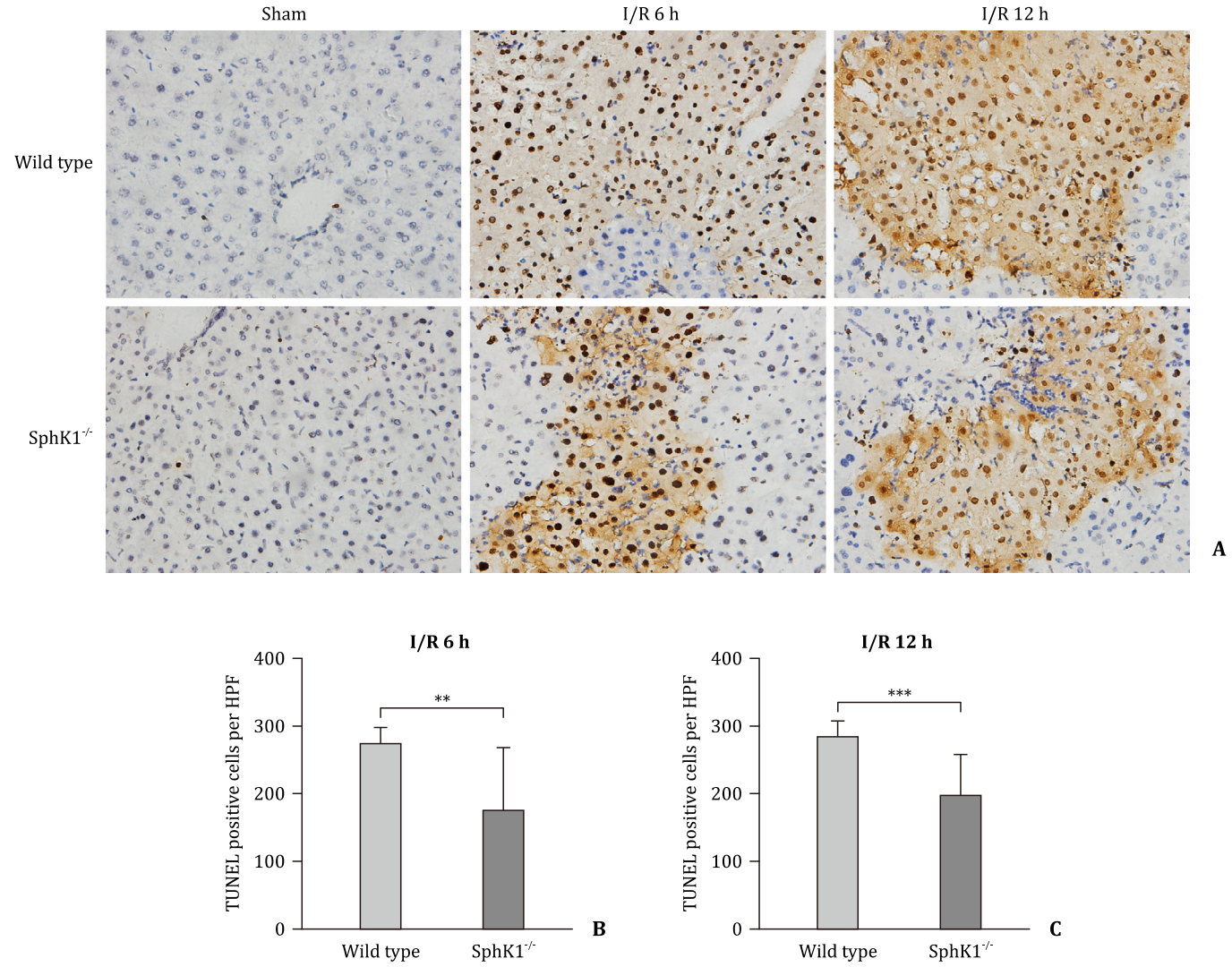

Fig. 3. TUNEL assay to determine hepatocyte apoptosis. **A:** TUNEL staining shows apoptotic hepatocytes after reperfusion of 6 h and 12 h ($n = 10$ for each group, light microscope, TUNEL staining, original magnification × 400); **B:** Determination of TUNEL positive cells per high power field (HPF) after reperfusion of 6 h ($n = 10$ for each group); **C:** Determination of TUNEL positive cells per HPF after reperfusion of 12 h ($n = 10$ for each group). **: $P < 0.01$; ***: $P < 0.001$. I/R: ischemia/reperfusion.

Fig. 6D). Taken together, these results suggested that the deletion of SphK1 inhibited I/R-induced liver inflammation.

SphK1 knockout improved I/R- induced oxidative stress

Fig. 7A showed that at 6 h of reperfusion, MPO level in wild type mice liver was elevated while the level of MPO in SphK1 knockout mice liver was significantly lower than that of wild type mice ($P < 0.001$). At 12 h of reperfusion, the level of MPO in both groups was decreased to a relatively lower level with no statistical difference between two groups. Level of MDA was significantly higher after I/R injury in both wild type and knockout groups compared with that in the sham operation groups. SphK1 knockout significantly decreased MDA at 6 h and 12 h of reperfusion compared with those of wild type mice liver tissues ($P < 0.05$ for both 6 h and 12 h, Fig. 7B).

Fig. 7C showed that although I/R insult caused obvious decrease of GSH content in liver tissue, it remained relatively higher than wild type in SphK1 knockout group ($P < 0.01$ for both 6 h and 12 h, Fig. 7C). Accordingly, GSH related antioxidant enzymes GSH-Px ($P < 0.001$ for 6 h, $P < 0.01$ for 12 h, Fig. 7D) and GST (Fig. 7F, $P < 0.001$ for 6 h, $P < 0.05$ for 12 h) showed similar trend during the course of I/R injury whereas GR showed no significant difference between the wild type and SphK1 knockout groups (Fig. 7E). Similarly, another antioxidant enzyme SOD also showed significantly higher level in the SphK1 deletion group compared with that in the wild type group at 6 h after reperfusion ($P < 0.01$, Fig. 7G). The activity of SOD at 12 h showed no difference between groups. H_2O_2 was significantly increased after reperfusion of 6 h and gradually decreased at 12 h. Fig. 7H indicated that the level of H_2O_2 was lower in the SphK1 knockout group than that in the wild type group at 6 h after reperfusion ($P < 0.01$).The catalase activities were significantly higher in SphK1 deletion liver at both 6 h ($P < 0.001$, Fig. 7I) and 12 h ($P < 0.05$, Fig. 7I) after reperfusion.

Fig. 7J showed that the content of NO was significantly higher in the wild type group than that in the SphK1 knockout group at 6 h after reperfusion ($P < 0.01$). Total NOS activity ($P < 0.05$, Fig. 7K) and iNOS activity ($P < 0.001$, Fig. 7L) were significantly increased in I/R injury compared with sham mice, while SphK1 deletion inhibited the NOS activities at 6 h after reperfusion but not at 12 h.

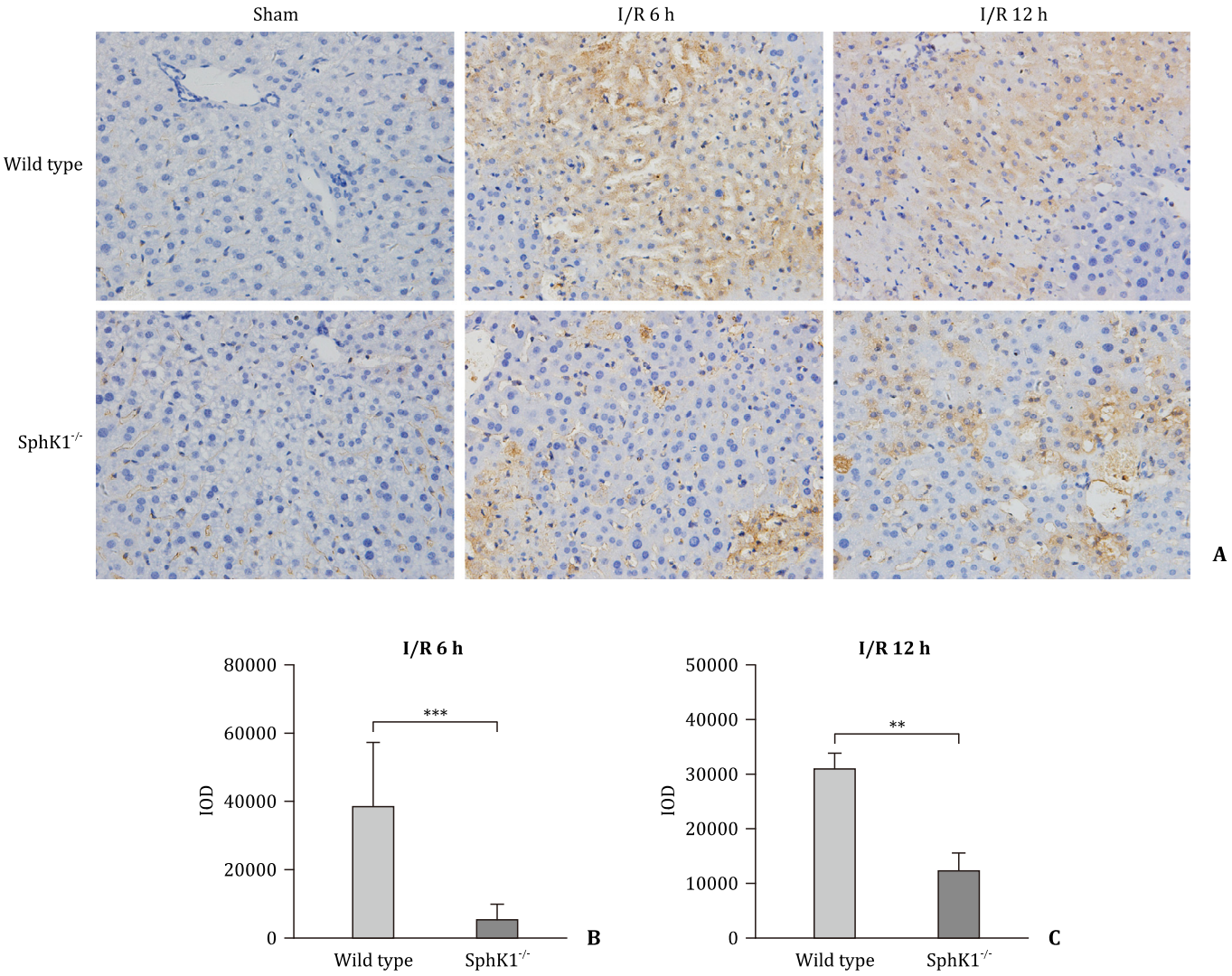

Fig. 4. Immunohistochemistry staining for phosphorylated STAT3. **A:** Immunostaining staining of phosphorylated STAT3 after reperfusion of 6 h and 12 h ($n = 10$ for each group, light microscope, DAB staining, original magnification × 400); **B:** Quantitative measurement of integrative optical density (IOD) of phosphorylated STAT3 at 6 h after reperfusion ($n = 10$ for each group); **C:** Quantitative measurement of IOD of phosphorylated STAT3 at 12 h after reperfusion ($n = 10$ for each group). **: $P < 0.01$; ***: $P < 0.001$. DAB: diaminobenzidine; STAT3: signal transducer and activator of transcription 3.

Discussion

As a bioactive metabolite of sphingolipid, S1P is a crucial second messenger that regulates a variety of physiologic and pathologic process including cell proliferation, survival, differentiation, immune reaction, tissue injury/repair and inflammation [6,12]. S1P is produced by two subtypes of kinases, SphK1 and SphK2, in mammals. A series of cytokines, growth factors and tissue injury/stress elevate the activity of sphingosine kinases and lead to the release of S1P [12,26,27]. The role of sphingosine kinases, S1P and S1PRs in I/R injury is controversial. It is well documented that hypoxia induces the expression of sphingosine kinase [27,28]. In myocardial I/R injury, SphK2 knockout sensitizes mice to heart damage and diminishes responsiveness to preconditioning. However, inhibition of sphingosine kinase by chemical inhibitor protects against myocardial I/R injury [29]. In renal I/R injury, SphK1 knockout mice had no alteration in renal function after I/R insult whereas constitutive expression of SphK2 may reduce I/R injury of the kidney [17]. For hepatic I/R injury, it has shown that the inhibition of SphK2 improves liver damage, possibly through the improvement of mitochondrial function and alleviation of inflammation [12,18]. Furthermore, FTY720, a small molecular inhibitor of S1P signaling, attenuates liver injury after transplantation [30]. These inconsistent results indicate that the role of S1P in I/R injury may be organ, kinase and receptor specific and the effect of SphK1 in hepatic I/R injury remains to be elucidated. Another possible reason for the inconsistency could be off target effects brought by RNA interference, small molecular inhibitors and other manipulative methods used in experiments. Studies using gene knockout animals may have the advantage of less confounding factors and may produce more reliable results. However, the effect of SphK1 in liver I/R injury has not been investigated using knockout animal models.

In this study, we investigated the role of SphK1 in liver partial warm I/R injury using knockout mice. After genotyping, the deletion of SphK1 in liver tissue were confirmed. We found that the expression of SphK1 was significantly increased after I/R insult in wild type mice, indicating this kinase may play a role in hepatic I/R injury. Serum ALT level is considered to be a classic indicator of hepatocellular damage. This study showed that hepatic I/R caused notable liver injury, which was maximized at 6 h after reperfusion and gradually recovered. Based on previous study [25], our preliminary exploratory experiments (data not

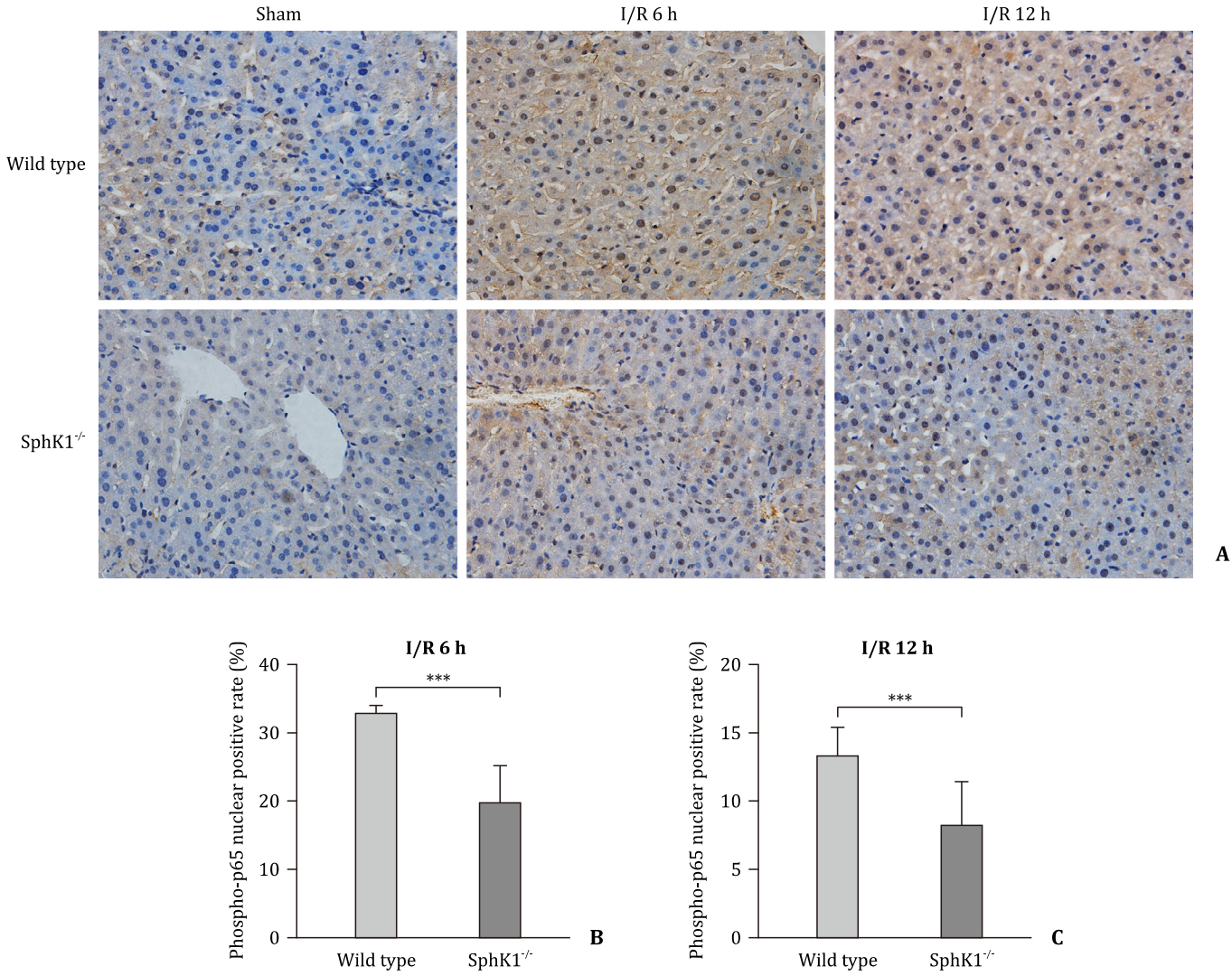

Fig. 5. Immunohistochemistry staining for phosphorylated p65. **A:** Immunostaining staining of phosphorylated p65 at 6 h and 12 h after reperfusion ($n = 10$ for each group, light microscope, DAB staining, original magnification × 400); **B:** Determination of phosphorylated p65 nuclear positive rate at 6 h after reperfusion ($n = 10$ for each group); **C:** Determination of phosphorylated p65 nuclear positive rate at 12 h after reperfusion ($n = 10$ for each group). ***: $P < 0.001$. DAB: diaminobenzidine.

shown) and current presented data, the hepatic damage, inflammatory reaction and oxidative stress could reach a peak at 6 h after reperfusion. Moreover, our results showed that the expression of SphK1 remained a high level at 12 h after reperfusion, indicating SphK1 could have prolonged effect even after the peak of injury in liver I/R. Taken together, 6 h and 12 h after reperfusion were chosen as critical and representative time points in the following experiments. Interestingly, genetic deletion of SphK1 significantly attenuated the degree of liver injury after I/R at both 6 h and 12 h after reperfusion. Histopathologic studies also confirmed that SphK1 knockout significantly ameliorated hepatic necrosis and hepatocellular apoptosis after I/R. Our results provided the evidence that abolishment of the activity of SphK1 at genetic level improved hepatic damage following I/R insult.

We also sought to shed light on the potential mechanisms underlying the attenuation of I/R injury by SphK1 deletion with focusing on inflammation and oxidative stress, which are considered two major mechanisms causing tissue damage. Previous studies have identified a complex cascade of proinflammatory mediators involved in the pathogenesis of liver I/R injury [1–3,24]. After I/R insult, Kupffer cells, T cells and to a lesser extent, endothelial cells and hepatocytes were activated and toxic proinflammatory cytokines including IL-1β, TNF-α and IL-6 were secreted [3,4]. The expression of these proinflammatory cytokines is largely dependent on the activation of master transcription factors NF-κB and STAT3, which could be persistently activated through S1PR1 signaling [8]. Therefore, the induction of SphK1 during I/R injury may be an important initiator and maintainer of inflammation cascade through S1P and downstream NF-κB, STAT3 signaling. This study confirmed that the level of nuclear translocation and/or phosphorylation of NF-κB and STAT3 were significantly elevated after hepatic I/R insult. The activities of these transcription factors sustained evident even at 12 h after reperfusion. In accordance with the observed attenuated hepatic damage, knockout of SphK1 also potently ameliorated the hyperactivation of the two central inflammatory transcription factors and the induction of proinflammatory S1P receptor S1PR1. As a result of downregulation of inflammatory signaling, the production of cytokines including IL-1β, IL-6 and TNF-α, were also inhibited. These results suggest that deletion of SphK1 improved inflammation after hepatic I/R, possibly through reduced secretion of S1P, attenuated S1PR1-mediated NF-κB and STAT3 hyperactivation and finally decreased production of toxic proinflammatory cytokines.

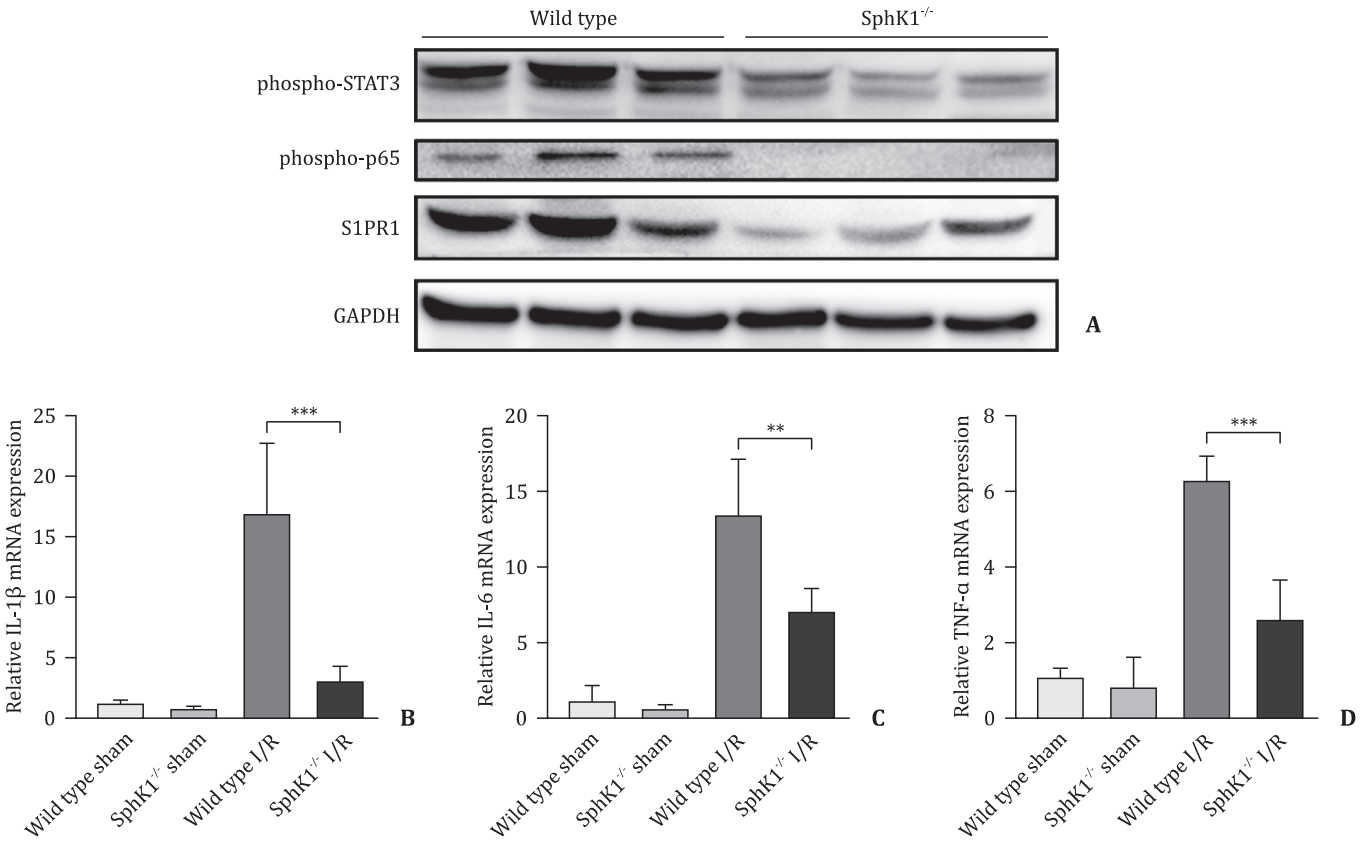

Fig. 6. Expression of phosphorylated STAT3, phosphorylated p65, S1PR1 and downstream proinflammatory cytokines. **A:** Western blotting detected the amount of phosphorylated STAT3, phosphorylated p65 and S1PR1 at 6 h after reperfusion. GAPDH served as loading control. Quantitative real-time PCR determined the expression of proinflammatory cytokines IL-1β (**B**), IL-6 (**C**) and TNF-α (**D**) (for real-time PCR experiments, $n=4$ for each group, these results are representative for three repeats of real-time PCR experiments). **: $P<0.01$; ***: $P<0.001$. STAT3: signal transducer and activator of transcription 3.

It is well established that the accumulation of ROS and disruption of redox homeostasis is closely correlated with the pathogenesis of I/R injury. The release of ROS, NO and other highly active free radicals mediates tissue damage after I/R [31]. Neutrophil is one of the earliest participators of I/R injury through the release of ROS, proteases and other mediators of tissue damage. As a specific intracellular content of neutrophil, MPO was used to assess the accumulation of neutrophil. We found that the MPO activity rapidly increased after reperfusion in parallel with the increase of ALT levels. This elevation of MPO was also inhibited by SphK1 knockout. ROS mediated lipid peroxidation after I/R insult was further evaluated by MDA. Like MPO accumulation, the concentration of MDA also increased in accordance with the time course of ALT levels. Similarly, the deletion of SphK1 also evidently attenuated the production of MDA after hepatic I/R. Under physiologic condition, ROS and other free radicals are rapidly detoxified by endogenous anti-oxidative enzymes and anti-oxidants. I/R injury is accompanied by the disruption of redox homeostasis, resulting in decreased ability of detoxification [1,31]. Overexpression or supplementation of anti-oxidative enzymes or antioxidants showed protective effect against I/R injury [32,33]. In the present study, the concentration of antioxidant GSH and the activity of anti-oxidative enzymes including GSH-Px, GST and SOD were significantly decreased after I/R. The knockout of SphK1 partially reversed the reduction of anti-oxidative capacity of the liver, which may be another underlying mechanism mediating the protective effect against I/R injury. As another oxidative toxin, H_2O_2 and its detoxifying enzyme CAT forms another couple in the maintenance of redox homeostasis. Hepatic I/R insult increased H_2O_2 and inhibited the activity of CAT, which are also partially reversed by SphK1 deletion. The role of NO in I/R injury remains controversial. Both protective and deleterious effect of NO are reported [34]. NO is synthesized by the three NOS, including endothelial NOS (eNOS), neuronal NOS (nNOS), and iNOS [35]. It is well established that NO concentrations during inflammatory statuses are significantly increased by iNOS [34]. In a rat model of hepatic I/R injury, iNOS inhibitor improved liver damage after reperfusion [36]. Another study also demonstrated that selective iNOS inhibitor attenuated liver damage following I/R, possibly through the inhibition of injury-provoking iNOS [37]. In our experiments, increased concentration of NO was observed in accordance with the elevation of iNOS and total NOS activities after reperfusion. Genetic deletion of SphK1 significantly inhibited both NO and NOS activity. Overall, our results indicated that SphK1 knockout improved oxidative stress following hepatic I/R insult. This partial restoration of redox homeostasis and its possible interaction with inflammatory reaction may represent another underlying mechanism of the attenuation of liver damage by SphK1 inhibition.

In summary, our present study demonstrated that knockout of SphK1 significantly alleviated tissue damage after hepatic I/R injury, possibly through inhibiting inflammation and oxidative stress. Our results have potential clinical importance since hepatic I/R injury is still a big event in surgical treatment of liver disease. It is well-known that liver I/R injury significantly increase the morbidity and mortality after hepatectomy and liver transplantation [2]. Recently, several studies revealed that I/R injury may even significantly promote the recurrence of hepatocellular carcinoma after liver resection [38,39] and liver transplantation [40]. The alleviation of I/R injury may not only diminish surgical morbidity but also reduce postoperative recurrence of liver malignancy [39].

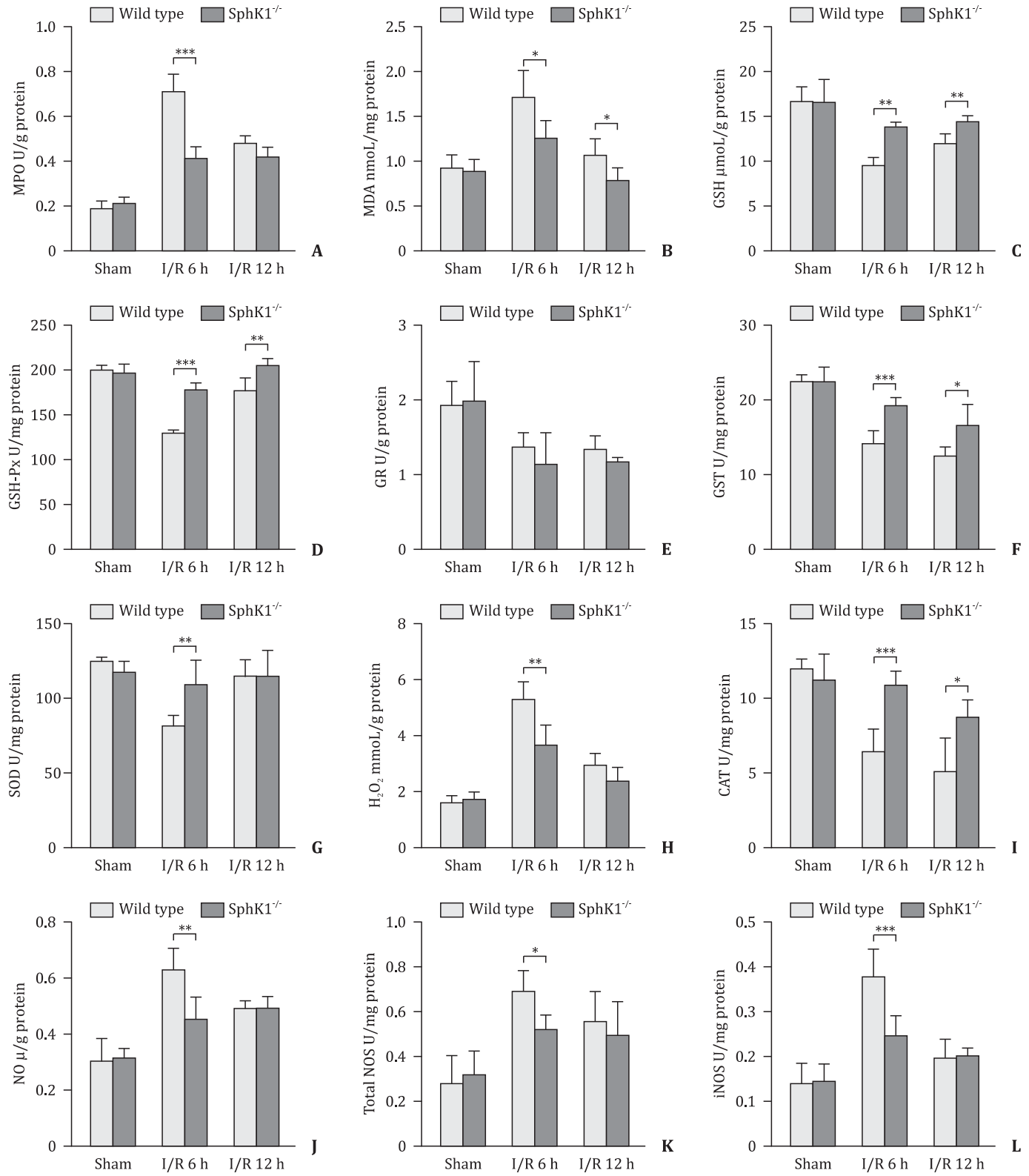

Fig. 7. Changes of oxidative markers after I/R insult. **A:** MPO; **B:** MDA; **C:** GSH; **D:** GSH-Px; **E:** GR; **F:** GST; **G:** SOD; **H:** H_2O_2; **I:** CAT; **J:** NO; **K:** total NOS activity; **L:** iNOS activity. $n = 5$ for each group. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. MPO: myeloperoxidase; MDA: malondialdehyde; GSH: glutathione; GSH-Px: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-S-transferase; SOD: superoxide dismutase; CAT: catalase; NO: nitric oxide; NOS: nitric oxide synthase; iNOS: inducible nitric oxide synthase.

Therefore, effective therapeutic target and efficient therapeutic approach against liver I/R injury is urgently needed. Our results indicated that SphK1 may represent a novel and potent target in hepatic I/R injury. Further investigations are needed to elucidate the mechanisms underlying the role of SphK1 in hepatic I/R injury. Since currently direct deletion or interference of SphK1 remains ethically controversial and technically difficult in clinical settings, studies to screen and validate SphK1 specific inhibitors for potential clinical application is also of significant importance.

Acknowledgments

We thank the staff of the Translational Medicine Core facilities of the Medical School of Nanjing University for instrument support.

Contributors

WZX, ZYY and JCP proposed the study. QGH, JAL and WJY performed the research. QGH, CY and ZG collected and analyzed the data. QGH and WZX wrote the manuscript. All authors contributed to the design and interpretation of the study and to further drafts. QGH and WZX contributed equally to this work. JCP is the guarantor.

Funding

This study was supported by grants from the National Natural Science Foundation of China (81572393 and 81602093), the Natural Science Foundation of Jiangsu Province (BK20160118), the Key Projects supported by Medical Science and Technology Development Foundation, Nanjing Municipality Health Bureau (ZKX15020 and ZKX17022), and Fundamental Research Funds for the Central Universities (021414380215, 020414380080 and 021414380329).

Ethical approval

This study was approved by the Ethics Committee of Drum Tower Clinical College of Nanjing Medical University.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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